

## HETEROCYCLIC SUBSTITUTED 1,4-DIHYDRO-4-OXO-1,8-NAPHTHPYRIDINE ANALOGS

### Cross-Reference to Related Applications

[0001] This application claims benefit of U.S. provisional application 60/461,205, filed April 7, 2003, and 60/519,569, filed November 12, 2003. The contents of these documents are incorporated herein by reference.

### Field of the Invention

[0002] The invention relates to substituted 1,4-dihydro-4-oxo-1,8-naphthpyridine analogs, and treatment methods using such compounds.

### Background

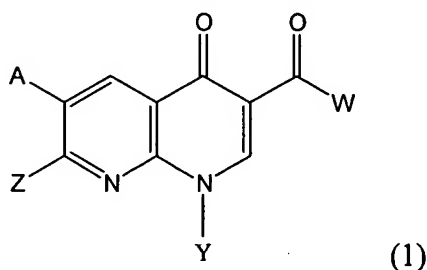
[0003] Quadruplexes can form in certain purine-rich strands of nucleic acids. In duplex nucleic acids, certain purine rich strands are capable of engaging in a slow equilibrium between a typical duplex helix structure and in unwound and non-B-form regions. These unwound and non-B forms can be referred to as "paranemic structures." Some forms are associated with sensitivity to S1 nuclease digestion, which can be referred to as "nuclease hypersensitivity elements" or "NHEs." A quadruplex is one type of paranemic structure and certain NHEs can adopt a quadruplex structure. Considerable circumstantial evidence suggests that quadruplex structures can exist in vivo in specific regions of the genome, including the telomeric ends of chromosomes and oncogene regulatory regions. (Han, *et al.*, *Trends Pharm. Sci.* (2000) 21:136-142). Thus, quadruplex forming regions of DNA may be used as molecular targets for anticancer agents.

### Summary of the Invention

[0004] Compounds described herein interact with regions of DNA that can form quadruplexes and act as tumor suppression genes with high affinity. Such compounds can reduce expression of highly proliferate genes and are utilized for treating cancers. Furthermore, the compounds may also exhibit antibacterial or antiviral activity, and may be used for treating bacterial and viral infections.

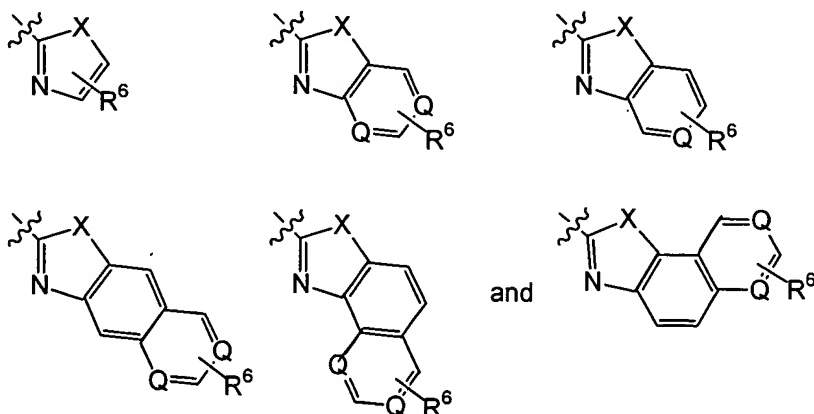
[0005] Various embodiments of the compounds of the present invention are described below. The present invention encompasses other compounds having formula 1, with substituents independently selected from compounds in Table 1. Thus, the present invention is not limited to the specific combination of substituents described in various embodiments below.

[0006] The compounds have the general formula:



and pharmaceutically acceptable salts, esters and prodrugs thereof;  
wherein W and Z are independently OR<sup>2</sup> or NR<sup>1</sup>R<sup>2</sup> wherein R<sup>1</sup> and R<sup>2</sup> may form an optionally substituted ring;  
A is H, halo or NR<sup>1</sup><sub>2</sub>;  
R<sup>1</sup> is H or a C<sub>1-6</sub> alkyl;  
R<sup>2</sup> is H or a C<sub>1-10</sub> alkyl or C<sub>2-10</sub> alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O, and S, and optionally substituted with a carbocyclic or heterocyclic ring; or R<sup>2</sup> is an optionally substituted heterocyclic ring, aryl or heteroaryl;

[0007] Y is selected from the group consisting of



where R<sup>6</sup> is a substituent at any position on the fused ring; and is H, OR<sup>1</sup>, C<sub>1-6</sub> alkyl, C<sub>2-6</sub> alkenyl, each optionally substituted by halo, =O or one or more heteroatoms; or R<sup>5</sup> is an inorganic substituent; or two adjacent R<sup>6</sup> is linked to obtain a 5-6 membered substituted or unsubstituted

carbocyclic or heterocyclic ring, optionally fused to an additional substituted or unsubstituted carbocyclic or heterocyclic ring;

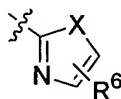
Q is CH or N;

and X is O, NH, or S;

provided that W is not hydroxy or ethoxy when Y is 2-thiazolyl or Z is 3-amino-1-pyrrolidinyl.

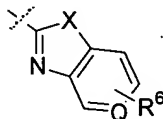
[0008] In the above formula 1, A may be halo. In one example, A is fluoro.

[0009] In the above formula 1, Y may have the formula



where X is S and R<sub>6</sub> is H;

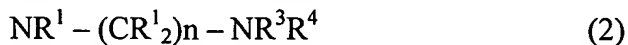
or the formula



where X is S, Q is CH, and R<sub>6</sub> is H.

[0010] In the above formula 1, W and Z may independently be NR<sup>1</sup>R<sup>2</sup>. In one example, R<sup>1</sup> is H and R<sup>2</sup> is a C<sub>1-10</sub> alkyl optionally containing one or more heteroatoms, and optionally substituted with a C<sub>3-6</sub> cycloalkyl, aryl or a 5-14 membered heterocyclic ring containing one or more N, O or S. In another example, R<sup>1</sup> is H and R<sup>2</sup> is an aryl or a 5-14 membered heterocyclic ring containing one or more N, O or S, each optionally substituted with an amino or another heterocyclic ring. In yet another example, R<sup>1</sup> and R<sup>2</sup> in NR<sup>1</sup>R<sup>2</sup> form an optionally substituted 5-14 membered ring containing one or more N, O or S. In particular examples, NR<sup>1</sup>R<sup>2</sup> is morpholine, thiomorpholine, piperazine, piperidine or diazepine.

[0011] In the above formula 1, W and Z may independently have the formula



wherein R<sup>1</sup> and R<sup>3</sup> are independently H or C<sub>1-6</sub> alkyl;

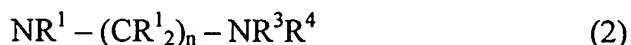
n is 1-6; and

$R^4$  is H or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; and

wherein in  $NR^3R^4$ ,  $R^3$  and  $R^4$  may form an optionally substituted ring.

[0012] In the above formula 2, n may be 2-3. In one example,  $NR^3R^4$  is an acyclic amine, or guanidinyll or a tautomer thereof; or  $R^3$  and  $R^4$  optionally form a substituted ring containing one or more N, O or S. In particular examples,  $NR^3R^4$  is morpholine, thiomorpholine, imidazole, pyrrolidine, piperazine, pyridine or piperidine.

[0013] In the above formula 1, Z may be  $NR^1R^2$ ; and W may have the formula



wherein  $R^1$  and  $R^2$  are as defined in claim 1;

$R^3$  is H or  $C_{1-6}$  alkyl;

n is 1-6; and

$R^4$  is H or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; and

wherein in  $NR^1R^2$  and  $NR^3R^4$ ,  $R^1$  and  $R^2$ , and  $R^3$  and  $R^4$  each independently may form a substituted ring.

[0014] In the above formula 2, where Z is  $NR^1R^2$ ; and W has the formula  $NR^1 - (CR^1_2)_n - NR^3R^4$  (2),  $R^1$  and  $R^2$  in  $NR^1R^2$ , and  $R^3$  and  $R^4$  in  $NR^3R^4$  each may independently form a substituted ring containing one or more N, O or S. For example, Z is optionally substituted with amino, carbamate, a  $C_{1-10}$  alkyl containing one or more non-adjacent N, O or S, and optionally substituted with a heterocyclic ring; aryl or a saturated or unsaturated heterocyclic ring, each of which is optionally substituted. In one example, Z and  $NR^3R^4$  are independently morpholine, thiomorpholine, imidazole, pyrrolidine, piperazine, pyridine or piperidine. In one example, Z and  $NR^3R^4$  are independently pyrrolidine. In another example, Z is pyrrolidine substituted with pyrazine.

[0015] Examples of 5-6 membered heterocyclic rings include but are not limited to tetrahydrofuran, 1,3-dioxolane, 2,3-dihydrofuran, tetrahydropyran, benzofuran, isobenzofuran, 1,3-dihydro-isobenzofuran, isoxazole, 4,5-dihydroisoxazole, piperidine, pyrrolidine,

pyrrolidin-2-one, pyrrole, pyridine, pyrimidine, octahydro-pyrrolo[3,4-*b*]pyridine, piperazine, pyrazine, morpholine, thiomorpholine, imidazole, imidazolidine-2,4-dione, benzimidazole, 1,3-dihydrobenzimidazol-2-one, indole, thiazole, benzothiazole, thiadiazole, thiophene, tetrahydro-thiophene 1,1-dioxide, diazepine, triazole, guanidine, diazabicyclo[2.2.1]heptane, 2,5-diazabicyclo[2.2.1]heptane, and 2,3,4,4a,9,9a-hexahydro-1H- $\beta$ -carboline.

[0016] In the above formula 1, Z may be OR<sup>2</sup> and R<sup>2</sup> is a C<sub>1-6</sub> alkyl optionally substituted with a carbocyclic or heterocyclic ring.

[0017] In the above formula 1, each optionally substituted moiety is substituted with one or more halo, OR<sup>2</sup>, NR<sup>1</sup>R<sup>2</sup>, carbamate, C<sub>1-10</sub> alkyl, C<sub>2-10</sub> alkenyl, each optionally substituted by halo, =O, aryl or one or more heteroatoms; inorganic substituents, aryl, carbocyclic or a heterocyclic ring.

[0018] The compounds of the present invention may be chiral. As used herein, a chiral compound is a compound that is different from its mirror image, and has an enantiomer. Methods of synthesizing chiral compounds and resolving a racemic mixture of enantiomers are well known to those skilled in the art. See, *e.g.*, March, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, (1985), which is incorporated herein by reference.

[0019] The present invention also provides pharmaceutical compositions comprising compounds having formula 1, and a pharmaceutically acceptable excipient.

[0020] The present invention also provides methods for identifying a compound that interacts with a quadruplex-forming region of DNA, comprising

- a) contacting a nucleic acid capable of forming a quadruplex with a primer comprising a label to form a complex;
- b) contacting said complex with one or more test compounds and a polymerase to form a reaction mixture, and
- c) separating said reaction mixture by capillary electrophoresis to obtain one or more reaction products; and
- d) determining the extent of primer extension in said one or more reaction products.

[0021] In one example, the method further comprises the step of determining the binding affinity of said one or more test compounds for said nucleic acid. In a particular example, the label is a fluorescent label.

[0022] Furthermore, the present invention provides methods for ameliorating a cell proliferative disorder, comprising administering to a subject in need thereof an effective amount of a compound having formula 1 or a pharmaceutical composition thereof, thereby ameliorating said cell-proliferative disorder. In one example, the cell proliferative disorder is cancer. In another example, cell proliferation is reduced, or cell death is induced. The subject may be human or animal.

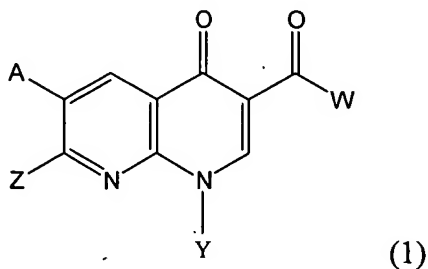
[0023] The present invention also provides methods for reducing cell proliferation or inducing cell death, comprising contacting a system with an effective amount of a compound having formula 1 or a pharmaceutical composition thereof, thereby reducing cell proliferation or inducing cell death in said system. The system may be a cell or tissue.

[0024] The present invention further provides methods for reducing microbial titers, comprising contacting a system with an effective amount of a compound having formula 1 or a pharmaceutical composition thereof, thereby reducing microbial titers. The system may be a cell or tissue. In one example, the microbial titers are viral, bacterial or fungal titers.

[0025] Further, the present invention provides methods for ameliorating a microbial infection, comprising administering to a subject in need thereof an effective amount of a compound having formula 1 or a pharmaceutical composition thereof, thereby ameliorating said microbial infection. The subject may be human or animal. In one example, the microbial infection is viral, bacterial or fungal.

#### Description of the Invention

[0026] The present invention relates to 1,4-dihydro-4-oxo-1,8-naphthpyridine analogs having formula I,



and pharmaceutically acceptable salts, esters, and prodrugs thereof.

[0027] In particular embodiments, the compounds interact with regions of DNA that can form quadruplexes. The present invention also relates to methods for treating cancer, bacterial and viral infections using such compounds.

[0028] Because regions of DNA that can form quadruplexes are regulators of biological processes such as oncogene transcription, modulators of quadruplex biological activity can be utilized as cancer therapeutics. Molecules that interact with regions of DNA that can form quadruplexes can exert a therapeutic effect on certain cell proliferative disorders and related conditions. Particularly, abnormally increased oncogene expression can cause cell proliferative disorders and quadruplex structures typically down-regulate oncogene expression. Examples of oncogenes include but are not limited to MYC, HIF, VEGF, ABL, TGF, PDGFA, MYB, SPARC, HUMTEL, HER, VAV, RET, H-RAS, EGF, SRC, BCL1, BCL2, and other oncogenes known to one of skill in the art.

[0029] Molecules that bind to regions of DNA that can form quadruplexes can exert a biological effect according to different mechanisms, which include for example, stabilizing a native quadruplex structure, inhibiting conversion of a native quadruplex to duplex DNA by blocking strand cleavage, and stabilizing a native quadruplex structure having a quadruplex-destabilizing nucleotide substitution and other sequence specific interactions. Thus, compounds that bind to regions of DNA that can form quadruplexes described herein may be administered to cells, tissues, or organisms for the purpose of down-regulating oncogene transcription and thereby treating cell proliferative disorders. The terms “treatment” and “therapeutic effect” as used herein refer to reducing or stopping a cell proliferation rate (*e.g.*, slowing or halting tumor growth) or reducing the number of proliferating cancer cells (*e.g.*, removing part or all of a tumor).

[0030] Determining whether the biological activity of native DNA that can form quadruplexes is modulated in a cell, tissue, or organism can be accomplished by monitoring quadruplex biological activity. Quadruplex forming regions of DNA biological activity may be monitored in cells, tissues, or organisms, for example, by detecting a decrease or increase of gene transcription in response to contacting the quadruplex forming DNA with a molecule. Transcription can be detected by directly observing RNA transcripts or observing polypeptides translated by transcripts, which are methods well known in the art.

[0031] Molecules that interact with quadruplex forming DNA and quadruplex forming nucleic acids can be utilized to treat many cell proliferative disorders. Cell proliferative disorders include, for example, colorectal cancers and hematopoietic neoplastic disorders (*i.e.*, diseases involving hyperplastic/neoplastic cells of hematopoietic origin such as those arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof). The diseases can arise from poorly differentiated acute leukemias, *e.g.*, erythroblastic leukemia and acute megakaryoblastic leukemia. Additional myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (Vaickus, Crit. Rev. in *Oncol./Hematol.* 11:267-297 (1991)). Lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGL), Hodgkin's disease and Reed-Sternberg disease. Cell proliferative disorders also include cancers of the colorectum, breast, lung, liver, pancreas, lymph node, colon, prostate, brain, head and neck, skin, liver, kidney, and heart. Compounds that interact with regions of DNA that can form quadruplexes also can be utilized to target cancer related processes and conditions, such as increased angiogenesis, by inhibiting angiogenesis in a subject.

[0032] The present invention provides a method for reducing cell proliferation or for treating or alleviating cell proliferative disorders, comprising contacting a system having a native DNA capable of forming a quadruplex region with a compound having formula I. The system may be a group of cells or one or more tissues. In one embodiment, the system is a subject in need of a treatment of a cell proliferative disorder (*e.g.*, a mammal such as a mouse, rat, monkey, or human). The present invention also provides a method for treating colorectal cancer by administering a compound that interacts with a c-MYC quadruplex forming region to a subject in need thereof, thereby reducing the colorectal cancer cell proliferation. Furthermore, the present invention provides a method for inhibiting angiogenesis and optionally treating a cancer associated with angiogenesis, comprising administering a compound that interacts with a vascular endothelial



growth factor (VEGF) quadruplex forming region to a subject in need thereof, thereby reducing angiogenesis and optionally treating a cancer associated with angiogenesis.

[0033] As used herein, the terms “treat,” “treatment” and “therapeutic effect” refer to reducing or stopping a cell proliferation rate (*e.g.*, slowing or halting tumor growth) or reducing the number of proliferating cancer cells (*e.g.*, removing part or all of a tumor). These terms also are applicable to reducing a titre of a microorganism in a system (*i.e.*, cell, tissue, or subject) infected with a microorganism, reducing the rate of microbial propagation, reducing the number of symptoms or an effect of a symptom associated with the microbial infection, and/or removing detectable amounts of the microbe from the system. Examples of microorganism include but are not limited to virus, bacterium and fungus.

[0034] Compounds that interact with quadruplex forming regions of DNA can also be used to reduce a microbial infection, such as a viral infection. Retroviruses offer a wealth of potential targets for G-quadruplex targeted therapeutics. G-quadruplex structures have been implicated as functional elements in at least two secondary structures formed by either viral RNA or DNA in HIV, the dimer linker structure (DLS) and the central DNA flap (CDF). Additionally, DNA aptamers which are able to adopt either inter- or intramolecular quadruplex structures are able to inhibit viral replication. In one example, DNA aptamers are able to inhibit viral replication by targeting the envelope glycoprotein (putatively). In another example, DNA aptamers inhibit viral replication by targeting the HIV-integrase respectively, suggesting the involvement of native quadruplex structures in interaction with the integrase enzyme.

[0035] Dimer linker structures, which are common to all retroviruses, serve to bind two copies of the viral genome together by a non-covalent interaction between the two 5' ends of the two viral RNA sequences. The genomic dimer is stably associated with the gag protein in the mature virus particle. In the case of HIV, the origin of this non-covalent binding may be traced to a 98 base-pair sequence containing several runs of at least two consecutive guanines (*e.g.*, the 3' for the formation of RNA dimers *in vitro*). An observed cation (potassium) dependence for the formation and stability of the dimer *in vitro*, in addition to the failure of an antisense sequence to effectively dimerize, has revealed the most likely binding structure to be an intermolecular G-quadruplex.

[0036] Prior to integration into the host genome, reverse transcribed viral DNA forms a pre-integration complex (PIC) with at least two major viral proteins, integrase and reverse

transcriptase, which is subsequently transported into the nucleus. The Central DNA Flap (CDF) refers to 99-base length single-stranded tail of the + strand, occurring near the center of the viral duplex DNA, which is known to play a role in the nuclear import of the PIC. Oligonucleotide mimics of the CDF have been shown to form intermolecular G-quadruplex structures in cell-free systems.

[0037] Thus, compounds that recognize quadruplex forming regions can be used to stabilize the dimer linker structure and thus prevent de-coupling of the two RNA strands. Also, by binding to the quadruplex structure formed by the CDF, protein recognition and/or binding events for nuclear transport of the PIC may be disrupted. In either case, a substantial advantage can exist over other anti-viral therapeutics. Current Highly Active Anti-Retroviral Therapeutic (HAART) regimes rely on the use of combinations of drugs targeted towards the HIV protease and HIV integrase. The requirement for multi-drug regimes is to minimize the emergence of resistance, which will usually develop rapidly when agents are used in isolation. The source of such rapid resistance is the infidelity of the reverse transcriptase enzyme which makes a mutation approximately once in every 10,000 base pairs. An advantage of targeting viral quadruplex structures over protein targets, is that the development of resistance is slow or is impossible. A point mutation of the target quadruplex can compromise the integrity of the quadruplex structure and lead to a non-functional copy of the virus. A single therapeutic agent based on this concept may replace the multiple drug regimes currently employed, with the concomitant benefits of reduced costs and the elimination of harmful drug/drug interactions.

[0038] The present invention provides a method for reducing a microbial titer in a system, comprising contacting a system having a native DNA quadruplex forming region with a compound having formula I. The system may be one or more cells or tissues. Examples of microbial titers include but are not limited to viral, bacterial or fungal titers. In a particular embodiment, the system is a subject in need of a treatment for a viral infection (*e.g.*, a mammal such as a mouse, rat, monkey, or human). Examples of viral infections include infections by a hepatitis virus (*e.g.*, hepatitis B or C), human immunodeficiency virus (HIV), rhinovirus, herpes-zoster virus (VZV), herpes simplex virus (*e.g.*, HSV-1 or HSV-2), cytomegalovirus (CMV), vaccinia virus, influenza virus, encephalitis virus, hantavirus, arbovirus, West Nile virus, human papilloma virus (HPV), Epstein-Barr virus, and respiratory syncytial virus. The present invention also provides a method

for treating HIV infection by administering a compound having formula I to a subject in need thereof, thereby reducing the HIV infection.

Identifying compounds that can bind to quadruplex forming regions of DNA

[0039] Compounds described herein are identified as compounds that can bind to quadruplex forming regions of DNA where a biological activity of this region, often expressed as a “signal,” produced in a system containing the compound is different than the signal produced in a system not containing the compound. While background signals may be assessed each time a new molecule is probed by the assay, detecting the background signal is not required each time a new molecule is assayed.

[0040] Examples of quadruplex forming nucleotide sequences are set forth in the following Table A:

<u>SEQUENCE</u>	<u>SEQ ID NO</u>	<u>ORIGIN</u>
TG <sub>4</sub> AG <sub>3</sub> TG <sub>4</sub> AG <sub>3</sub> TG <sub>4</sub> AAGG	1	CMYC
GGGGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGGC	2	PDGFA
G <sub>8</sub> ACGCG <sub>3</sub> AGCTG <sub>5</sub> AG <sub>3</sub> CTTG <sub>4</sub> CCAG <sub>3</sub> CG <sub>4</sub> CGCTTAG <sub>5</sub>	3	PDGFB/c -sis
AGGAAGGGGAGGGCCGGGGGGAGGTGGC	4	CABL
AGGGGCGGGGCGGGGCGGGGGC	5	RET
GGGAGGAAGGGGGCGGGAGCGGGGC	6	BCL-2
GGGGGGCGGGGGCGGGCGCAGGGGGAGGGGGC	7	Cyclin D1/BCL-1
CGGGGCGGGGCGGGGGCGGGGGC	8	H-RAS
AGAGGAGGAGGAGGTACGGAGGAGGAGGAGAAGGAGGAGGAGGAA	9	CMYB
(GGA) <sub>4</sub>	10	VAV
AGAGAAGAGGGGAGGAGGAGGAGGAGGAGGAGGAGGCGC	11	HMGA2
GGAGGGGGAGGGG	12	CPIM
AGGAGAAGGAGGAGGTGGAGGAGGAGG	13	HER2/neu
AGGAGGAGGAGAATGCGAGGAGGAGGGAGGAGA	14	EGFR
GGGGCGGGCCGGGGGGCGGGTCCCGGCGGGGCGGAG	15	VEGF
CGGGAGGAGGAGGAAGGAGGAAGCGCG	16	CSRC

[0041] In addition to determining whether a test molecule or test nucleic acid gives rise to a different signal, the affinity of the interaction between the nucleic acid and the compound may be quantified.  $IC_{50}$ ,  $K_d$ , or  $K_i$  threshold values may be compared to the measured  $IC_{50}$  or  $K_d$  values for each interaction, and thereby identify a test molecule as a quadruplex interacting molecule or a test nucleic acid as a quadruplex forming nucleic acid. For example,  $IC_{50}$  or  $K_d$  threshold values of 10  $\mu$ M or less, 1  $\mu$ M or less, and 100 nM or less are often utilized. In another example, threshold values of 10 nM or less, 1 nM or less, 100 pM or less, and 10 pM or less may be utilized to identify quadruplex interacting molecules and quadruplex forming nucleic acids.

[0042] Many assays are available for identifying compounds that have affinity for quadruplex forming regions of DNA. In some of these assays, the biological activity is the quadruplex nucleic acid binding to a compound and binding is measured as a signal. In other assays, the biological activity is a polymerase arresting function of a quadruplex and the degree of arrest is measured as a decrease in a signal. In certain assays, the biological activity is transcription and transcription levels can be quantified as a signal. In another assay, the biological activity is cell death and the number of cells undergoing cell death is quantified. Another assay monitors proliferation rates of cancer cells. Examples of assays are fluorescence binding assays, gel mobility shift assays (see, *e.g.*, Jin & Pike, *Mol. Endocrinol.* (1996) 10:196-205), polymerase arrest assays, transcription reporter assays, cancer cell proliferation assays, and apoptosis assays (see, *e.g.*, Amersham Biosciences (Piscataway, New Jersey)), and embodiments of such assays are described hereafter. Also, topoisomerase assays can be utilized to determine whether the quadruplex interacting molecules have a topoisomerase pathway activity (see, *e.g.*, TopoGEN, Inc. (Columbus, Ohio)).

#### Gel Electrophoretic Mobility Shift Assay (EMSA)

[0043] An EMSA is useful for determining whether a nucleic acid forms a quadruplex and whether a nucleotide sequence is quadruplex-destabilizing. EMSA is conducted as described previously (Jin & Pike, *Mol. Endocrinol.* 10: 196-205 (1996)) with minor modifications. Generally, synthetic single-stranded oligonucleotides are labeled in the 5' -terminus with T4-kinase in the presence of [ $\gamma$ - $^{32}$ P] ATP (1,000 mCi/mmol, Amersham Life Science) and purified through a sephadex column.  $^{32}$ P-labeled oligonucleotides (~30,000 cpm) are then incubated with or without

various concentrations of a testing compound in 20  $\mu$ l of a buffer containing 10 mM Tris pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 5 mM  $MgCl_2$ , 10% glycerol, 0.05% Nonidet P-40, and 0.1 mg/ml of poly(dI-dC) (Pharmacia). After incubation for 20 minutes at room temperature, binding reactions are loaded on a 5% polyacrylamide gel in 0.25 x Tris borate-EDTA buffer (0.25 x TBE, 1 x TBE is 89 mM Tris-borate, pH 8.0, 1 mM EDTA). The gel is dried and each band is quantified using a phosphoimager.

#### DMS Methylation Protection Assay

[0044] Chemical footprinting assays are useful for assessing quadruplex structure. Quadruplex structure is assessed by determining which nucleotides in a nucleic acid are protected or unprotected from chemical modification as a result of being inaccessible or accessible, respectively, to the modifying reagent. A DMS methylation assay is an example of a chemical footprinting assay. In such an assay, bands from EMSA are isolated and subjected to DMS-induced strand cleavage. Each band of interest is excised from an electrophoretic mobility shift gel and soaked in 100 mM KCl solution (300  $\mu$ l) for 6 hours at 4°C. The solutions are filtered (microcentrifuge) and 30,000 cpm (per reaction) of DNA solution is diluted further with 100 mM KCl in 0.1X TE to a total volume of 70  $\mu$ l (per reaction). Following the addition of 1  $\mu$ l salmon sperm DNA (0.1  $\mu$ g/ $\mu$ l), the reaction mixture is incubated with 1  $\mu$ l DMS solution (DMS:ethanol; 4:1; v:v) for a period of time. Each reaction is quenched with 18  $\mu$ l of stop buffer (b-mercaptoethanol:water:NaOAc (3 M); 1:6:7; v:v:v). Following ethanol precipitation (twice) and piperidine cleavage, the reactions are separated on a preparative gel (16%) and visualized on a phosphoimager.

#### Polymerase Arrest Assay

[0045] An arrest assay includes a template nucleic acid, which may comprise a quadruplex forming sequence, and a primer nucleic acid which hybridizes to the template nucleic acid 5' of the quadruplex-forming sequence. The primer is extended by a polymerase (*e.g.*, Taq polymerase), which advances from the primer along the template nucleic acid. In this assay, a quadruplex structure can block or arrest the advance of the enzyme, leading to shorter transcription fragments. Also, the arrest assay may be conducted at a variety of temperatures, including 45°C and 60°C, and at a variety of ion concentrations.

[0046] An example of the Taq polymerase stop assay is described in Han, *et al.*, *Nucl. Acids Res.* (1999) 27:537-542, which is a modification of that used by Weitzmann, *et al.*, *J. Biol. Chem.* (1996) 271:20958–20964. Briefly, a reaction mixture of template DNA (50 nM), Tris-HCl (50 mM), MgCl<sub>2</sub> (10 mM), DTT (0.5 mM), EDTA (0.1 mM), BSA (60 ng), and 5'-end-labeled quadruplex nucleic acid (~18 nM) is heated to 90°C for 5 minutes and allowed to cool to ambient temperature over 30 minutes. Taq Polymerase (1 µl) is added to the reaction mixture, and the reaction is maintained at a constant temperature for 30 minutes. Following the addition of 10 µl stop buffer (formamide (20 ml), 1 M NaOH (200 µl), 0.5 M EDTA (400 µl), and 10 mg bromophenol blue), the reactions are separated on a preparative gel (12%) and visualized on a phosphoimager. Adenine sequencing (indicated by “A” at the top of the gel) is performed using double-stranded DNA Cycle Sequencing System from Life Technologies. The general sequence for the template strands is TCCAACATGTATAC-INSERT-TTAGCGACACGCAATTGCTATAGTGAGTCGTATTA, where “INSERT” refers to a nucleic acid sequence comprising a quadruplex forming sequence (See e.g., Table A). Bands on the gel that exhibit slower mobility are indicative of quadruplex formation.

#### High Throughput Polymerase Arrest Assay

[0047] A high throughput polymerase arrest assay has been developed. The assay comprises contacting a template nucleic acid, often DNA, with a primer, which also is often DNA; contacting the primer/template complex with a compound described herein (also referred to as a “test compound”); contacting the primer/template complex with a polymerase; and separating reaction products. The assay often includes the step of denaturing the primer/template complex mixture and then renaturing the complex, which often is carried out before a test molecule is added to the system. Multiple assays often are carried out using varying concentrations of a test compound, such that an IC<sub>50</sub> value can be obtained, for example. The reaction products often include extended primers of different lengths. Where a test compound does not significantly interact with a quadruplex structure in the template, the primer often is extended to the end of the template.

[0048] Where a test compound significantly interacts with a quadruplex structure in the template, the primer often is extended only to the quadruplex structure in the template and no further. Thus, the reaction mixture often includes at least two reaction products when a test

compound interacts with a quadruplex structure in the template, one having a completely extended primer and one having an incompletely extended primer, and these two reaction products are separated. The products may be separated using any convenient separation method, such as mass spectrometry and in one embodiment, capillary electrophoresis.

[0049] The reaction products often are identified by detecting a detectable label linked to the primer. The detectable label may be non-covalently linked to the 5' end of the primer (*e.g.*, a biotin molecule covalently linked to the 5' end of the primer which is non-covalently linked to an avidin molecule joined to a detectable label). The detectable label may be joined to the primer at any stage of the assay, sometimes before the primer is added to the system, after the primer is extended, or after the products are separated. The detectable label often is covalently linked to the primer using a procedure selected based upon the nature of the chemical groups in the detectable label.

[0050] Many methods for covalently linking detectable labels to nucleic acids are available, such as chemically coupling an allylamine-derivatized nucleotide to a succinimidyl-ester derivative of a detectable label, and then generating a primer using the labeled nucleotide. (See, *e.g.*, *Nature Biotech* (2000) 18:345-348 and [http address info.med.yale.edu/genetics/ward/tavi/n\\_coupling.html](http://info.med.yale.edu/genetics/ward/tavi/n_coupling.html)). A spacer (often between 5-16 carbon atoms long) sometimes is incorporated between the detectable label and the nucleotide. Any convenient detectable label may be utilized, including but not limited to a radioactive isotope (*e.g.*,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$  or  $^3\text{H}$ ); a light scattering label (*e.g.*, a spherical gold or silver label; Genicon Sciences Corporation, San Diego, CA and U.S. Patent No. 6,214,560); an enzymic or protein label (*e.g.*, GFP or peroxidase); or another chromogenic label or dye sometimes is utilized. Often, a fluorescent label is utilized (*e.g.*, amino-methyl coumarin (AMCA); diethyl aminomethyl coumarin (DEAC); cascade blue (CB); fluorescein isothiocyanate (FITC); Oregon green (OG); Alexa 488 (A488); rhodamine green (RGr); lanthanide chelate (*e.g.*, europium), carboxy-rhodamine 6G (R6G); tetramethyl rhodamine (TAMRA); Texas Red (TxR); Cy3; Cy3.5; Cy5, Cy5.5 and carboxynaphtofluorescein (CNF), digoxigenin (DIG); and 2,4-dinitrophenyl (DNP)). Other fluorophores and attendant excitation and emission wavelengths are described in Anantha, *et al.*, *Biochemistry* (1998) 37:2709-2714 and Qu & Chaires, *Methods Enzymol* (2000) 321:353-69).

[0051] In an embodiment, a primer oligonucleotide covalently linked to a fluorescent label is contacted with template DNA. The resulting complex is contacted with a test molecule and then

contacted with a polymerase capable of extending the primer. The reaction products then are separated and detected by capillary electrophoresis. A longer primer sequence was used for practicing this embodiment as compared to embodiments where the primer includes no covalently-linked fluorophore or where capillary electrophoresis is not utilized for separation. Deoxynucleotides are added at any stage of the assay before the separation, often when the primer is contacted with the template DNA. The template DNA/primer complex often is denatured (*e.g.*, by increasing the temperature of the system) and then renatured (*e.g.*, by cooling the system) before a test compound is added).

[0052] The following is a specific example of the assay embodiment. A 5'-fluorescent-labeled (FAM) primer (P45, 15 nM) was mixed with template DNA (15 nM) in a Tris-HCL buffer (15 mM Tris, pH 7.5) containing 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 0.1 mM mixed deoxynucleotide triphosphates (dNTP's). The FAM-P45 primer (5'-6FAM-AGTCTGAC TGACTGTACGTAGCTAATACGACTCACTATAGCAATT-3') and the template DNA (5'-TCCAACATATGTATACTGGGGAGGGTGGGGAGGGTGGGGAAGGTTAGCGACACGCA ATTGCTATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT-3') were synthesized and HPLC purified by Applied Biosystems. The mixture was denatured at 95°C for 5 minutes and, after cooling down to room temperature, was incubated at 37°C for 15 minutes.

[0053] After cooling down to room temperature, 1 mM KCl<sub>2</sub> and the test compound (various concentrations) were added and the mixture incubated for 15 minutes at room temperature. The primer extension was performed by adding 10 mM KCl and Taq DNA Polymerase (2.5 U/reaction, Promega) and incubating at 70°C for 30 minutes. The reaction was stopped by adding 1 µl of the reaction mixture to 10 µl Hi-Di Formamide mixed and 0.25 µl LIZ120 size standard. Hi-Di Formamide and LIZ120 size standard were purchased from Applied Biosystems. The partially extended quadruplex arrest product was between 61 or 62 bases long and the full-length extended product was 99 bases long. The products were separated and analyzed using capillary electrophoresis. Capillary electrophoresis was performed using an ABI PRISM 3100-Avant Genetic Analyzer. The assay was performed using compounds described above and results are shown in Table 1. µM concentrations reported in Table 1 are concentrations at which 50% of the DNA was arrested in the assay (*i.e.*, the ratio of shorter partially extended DNA (arrested DNA) to full-length extended DNA is 1:1).



#### Transcription Reporter Assay

[0054] In a transcription reporter assay, test quadruplex DNA is coupled to a reporter system, such that a formation or stabilization of a quadruplex structure can modulate a reporter signal. An example of such a system is a reporter expression system in which a polypeptide, such as luciferase or green fluorescent protein (GFP), is expressed by a gene operably linked to the potential quadruplex forming nucleic acid and expression of the polypeptide can be detected. As used herein, the term “operably linked” refers to a nucleotide sequence which is regulated by a sequence comprising the potential quadruplex forming nucleic acid. A sequence may be operably linked when it is on the same nucleic acid as the quadruplex DNA, or on a different nucleic acid. An exemplary luciferase reporter system is described herein.

[0055] A luciferase promoter assay described in He, *et al.*, *Science* (1998) 281:1509-1512 often is utilized for the study of quadruplex formation. Specifically, a vector utilized for the assay is set forth in reference 11 of the He, *et al.*, document. In this assay, HeLa cells are transfected using the lipofectamin 2000-based system (Invitrogen) according to the manufacturer’s protocol, using 0.1 µg of pRL-TK (Renilla luciferase reporter plasmid) and 0.9 µg of the quadruplex-forming plasmid. Firefly and Renilla luciferase activities are assayed using the Dual Luciferase Reporter Assay System (Promega) in a 96-well plate format according to the manufacturer’s protocol.

#### Circular Dichroism Assay

[0056] Circular dichroism (CD) is utilized to determine whether another molecule interacts with a quadruplex nucleic acid. CD is particularly useful for determining whether a PNA or PNA-peptide conjugate hybridizes with a quadruplex nucleic acid *in vitro*. PNA probes are added to quadruplex DNA (5 µM each) in a buffer containing 10 mM potassium phosphate (pH 7.2) and 10 or 250 mM KCl at 37°C and then allowed to stand for 5 minutes at the same temperature before recording spectra. CD spectra are recorded on a Jasco J- 715 spectropolarimeter equipped with a thermoelectrically controlled single cell holder. CD intensity normally is detected between 220 nm and 320 nm and comparative spectra for quadruplex DNA alone, PNA alone, and quadruplex DNA with PNA are generated to determine the presence or absence of an interaction (see, *e.g.*, Datta, *et al.*, *JACS* (2001) 123:9612-9619). Spectra are arranged to represent the average of eight scans recorded at 100 nm/min.

### Fluorescence Binding Assay

[0057] An example of a fluorescence binding assay is a system that includes a quadruplex nucleic acid, a signal molecule, and a test molecule. The signal molecule generates a fluorescent signal when bound to the quadruplex nucleic acid (*e.g.*, N-methylmesoporphyrin IX (NMM)), and the signal is altered when a test compound competes with the signal molecule for binding to the quadruplex nucleic acid. An alteration in the signal when test molecule is present as compared to when test compound is not present identifies the test compound as a quadruplex interacting compound.

[0058] 50  $\mu$ l of quadruplex nucleic acid or a nucleic acid not capable of forming a quadruplex is added in 96-well plate. A test compound also is added in varying concentrations. A typical assay is carried out in 100  $\mu$ l of 20 mM HEPES buffer, pH 7.0, 140 mM NaCl, and 100 mM KCl. 50  $\mu$ l of the signal molecule NMM then is added for a final concentration of 3  $\mu$ M. NMM is obtained from Frontier Scientific Inc, Logan, Utah. Fluorescence is measured at an excitation wavelength of 420 nm and an emission wavelength of 660 nm using a FluroStar 2000 fluorometer (BMG Labtechnologies, Durham, NC). Fluorescence often is plotted as a function of concentration of the test compound or quadruplex-targeted nucleic acid and maximum fluorescent signals for NMM are assessed in the absence of these molecules.

### Cell Proliferation Assay

[0059] In a cancer cell proliferation assay, cell proliferation rates are assessed as a function of different concentrations of test compounds added to the cell culture medium. Any cancer cell type can be utilized in the assay. In one embodiment, colon cancer cells are cultured *in vitro* and test compounds are added to the culture medium at varying concentrations. A useful colon cancer cell line is colo320, which is a colon adenocarcinoma cell line deposited with the National Institutes of Health as accession number JCRB0225. Parameters for using such cells are available at the [http address cellbank.nihs.go.jp/cell/data/jcrb0225.htm](http://cellbank.nihs.go.jp/cell/data/jcrb0225.htm).

### Formulation of Compounds

[0060] As used herein, the term "pharmaceutically acceptable salts, esters and amides" includes but are not limited to carboxylate salts, amino acid addition salts, esters and amides of the

compounds, as well as the zwitterionic forms thereof, which are known to those skilled in the art as suitable for use with humans and animals. (See, *e.g.*, Gerge, S.M., *et al.*, "Pharmaceutical Salts," *J. Pharm. Sci.* (1977) 66:1-19, which is incorporated herein by reference.)

[0061] Any suitable formulation of the compounds described herein can be prepared. In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids that form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts. Pharmaceutically acceptable salts are obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (*e.g.*, sodium, potassium or lithium) or alkaline earth metal (*e.g.*, calcium) salts of carboxylic acids also are made.

[0062] A compound may be formulated as a pharmaceutical composition and administered to a mammalian host in need of such treatment. In one embodiment, the mammalian host is human. Any suitable route of administration may be used, including but not limited to oral, parenteral, intravenous, intramuscular, topical and subcutaneous routes.

[0063] In one embodiment, a compound is administered systemically (*e.g.*, orally) in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, compressed into tablets, or incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

[0064] Tablets, troches, pills, capsules, and the like also may contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a

disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Any material used in preparing any unit dosage form is pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

[0065] The active compound also may be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in a buffered solution, often phosphate buffered saline, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The compound is sometimes prepared as a polymatrix-containing formulation for such administration (*e.g.*, a liposome or microsome). Liposomes are described for example in U.S. Patent No. 5,703,055 (Felgner, *et al.*) and Gregoriadis, *Liposome Technology* vols. I to III (2nd ed. 1993).

[0066] The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the particle size in the case of dispersions or

by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0067] Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

[0068] For topical administration, the present compounds may be applied in liquid form. Compounds often are administered as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid. Examples of useful dermatological compositions used to deliver compounds to the skin are known (see, *e.g.*, Jacquet, *et al.* (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith, *et al.* (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

[0069] Compounds may be formulated with a solid carrier, which include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers. Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

[0070] Generally, the concentration of the compound in a liquid composition often is from about 0.1 wt% to about 25 wt%, sometimes from about 0.5 wt% to about 10 wt%. The concentration in a semi-solid or solid composition such as a gel or a powder often is about 0.1 wt% to about 5 wt%, sometimes about 0.5 wt% to about 2.5 wt%. A compound composition may be prepared as a unit dosage form, which is prepared according to conventional techniques known in the pharmaceutical industry. In general terms, such techniques include bringing a compound into association with pharmaceutical carrier(s) and/or excipient(s) in liquid form or finely divided solid form, or both, and then shaping the product if required. The compound composition may be formulated into any dosage form, such as tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions also may be formulated as suspensions in aqueous, non-aqueous, or mixed media. Aqueous suspensions may further contain substances which increase viscosity, including for example, sodium carboxymethylcellulose, sorbitol, and/or dextran. The suspension may also contain one or more stabilizers.

[0071] The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

[0072] A useful compound dosage often is determined by assessing its *in vitro* activity in a cell or tissue system and/or *in vivo* activity in an animal system. For example, methods for extrapolating an effective dosage in mice and other animals to humans are known to the art (see, e.g., U.S. Pat. No. 4,938,949). Such systems can be used for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) of a compound. The dose ratio between a toxic and therapeutic effect is the therapeutic index and it can be expressed as the ratio ED<sub>50</sub>/LD<sub>50</sub>. The compound dosage often lies within a range of circulating concentrations for which the ED<sub>50</sub> is associated with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compounds used in the methods described herein, the therapeutically effective dose can be estimated initially from cell culture assays. A dose sometimes is formulated to achieve a circulating plasma concentration range covering the IC<sub>50</sub> (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as

determined in *in vitro* assays, as such information often is used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0073] Another example of effective dose determination for a subject is the ability to directly assay levels of “free” and “bound” compound in the serum of the test subject. Such assays may utilize antibody mimics and/or “biosensors” generated by molecular imprinting techniques. The compound is used as a template, or “imprinting molecule”, to spatially organize polymerizable monomers prior to their polymerization with catalytic reagents. Subsequent removal of the imprinted molecule leaves a polymer matrix which contains a repeated “negative image” of the compound and is able to selectively rebind the molecule under biological assay conditions (see, *e.g.*, Ansell, *et al.*, *Current Opinion in Biotechnology* (1996) 7:89-94 and in Shea, *Trends in Polymer Science* (1994) 2:166-173). Such “imprinted” affinity matrixes are amenable to ligand-binding assays, whereby the immobilized monoclonal antibody component is replaced by an appropriately imprinted matrix (see, *e.g.*, Vlatakis, *et al.*, *Nature* (1993) 361:645-647). Through the use of isotope-labeling, “free” concentration of compound can be readily monitored and used in calculations of  $IC_{50}$ . Such “imprinted” affinity matrixes can also be designed to include fluorescent groups whose photon-emitting properties measurably change upon local and selective binding of compound. These changes can be readily assayed in real time using appropriate fiberoptic devices, in turn allowing the dose in a test subject to be quickly optimized based on its individual  $IC_{50}$ . An example of such a “biosensor” is discussed in Kriz, *et al.*, *Analytical Chemistry* (1995) 67:2142-2144.

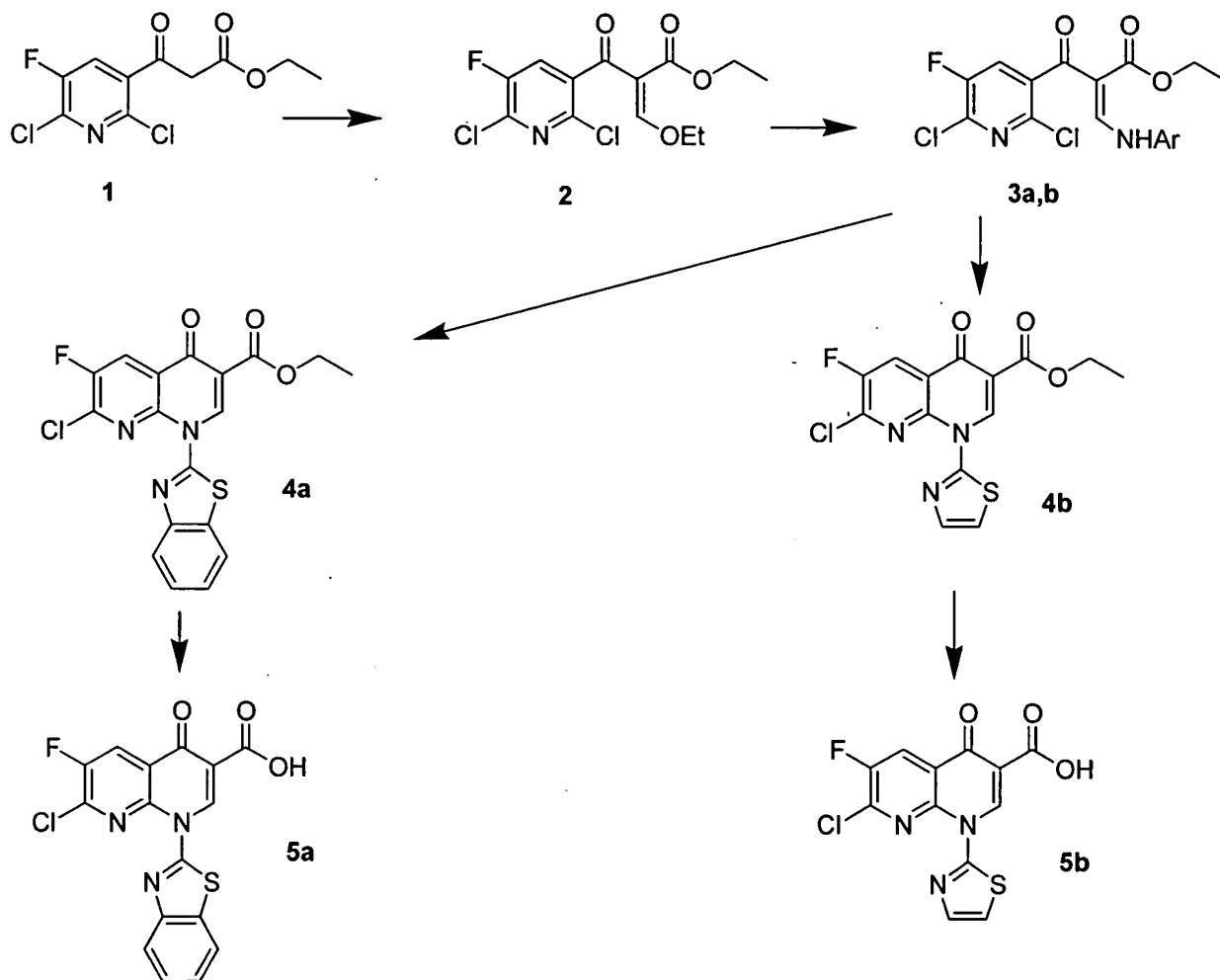
[0074] Exemplary doses include milligram or microgram amounts of the compound per kilogram of subject or sample weight, for example, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid described herein, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an

appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

[0075] The following examples are offered to illustrate but not to limit the invention.

### Examples

[0076] The following are exemplary procedures for synthesizing amide derivatives of heterocyclic substituted 1,4-dihydro-4-oxo1,8-naphthpyridine analogs.





Procedure

[0077] Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate (Aldrich) (2.0g, 5.9 mmol) was mixed with triethyl orthoformate (4.0 g) and acetic anhydride (2.0 g) and heated and stirred at 140°C for 45 minutes. The reaction was cooled and the resulting enol-ether solution (2) split into two equal halves.

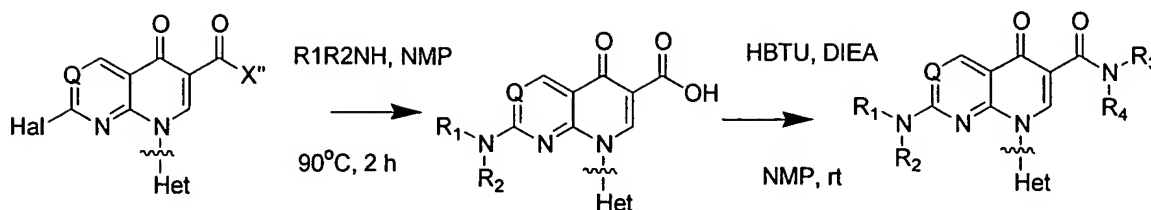
[0078] To the first half of the reaction, 2-aminothiazole (500 mg) in ethanol (5 ml) was added and the resulting solution was then stirred at room temperature for 1 h. The solution was then evaporated to a residue, dioxane (10 ml) and potassium carbonate (0.25 g) added and the mixture heated to reflux for 1 h. The resulting ethyl 7-chloro-6-fluoro-1,4-dihydro-4-oxo-1-(thiazol-2-yl)-1,8-naphthyridine-3-carboxylate was isolated by first filtering the mixture to remove the inorganic byproducts and the evaporation of the mother liquors.

[0079] The crude thiazole ester was dissolved in acetic acid (10 ml) and 12M HCl (10 ml) and heated to 50°C for 1.5 h. The resulting solution was cooled and partly evaporated until a solid formed and was collected. 7-chloro-6-fluoro-1,4-dihydro-4-oxo-1-(thiazol-2-yl)-1,8-naphthyridine-3-carboxylic acid (5b) was used without further purification.

[0080] To the second half of the enol-ether solution, 2-aminobenzothiazole (750 mg) in ethanol (5 ml) was added and the resulting solution was then stirred at room temperature for 1 h. The solution was then evaporated to a residue, dioxane (10 ml) and potassium carbonate (0.25 g) added and the mixture heated to reflux for 1 h. The resulting ethyl 1-(benzo[d]thiazol-2-yl)-7-chloro-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylate was isolated by first filtering the mixture to remove the inorganic byproducts and the evaporation of the mother liquors.

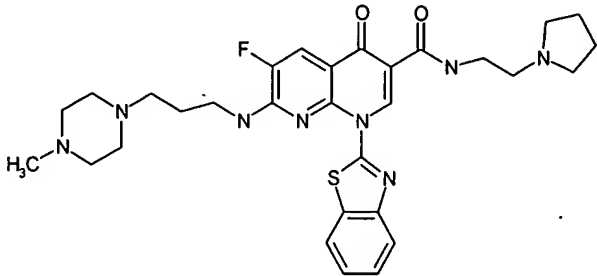
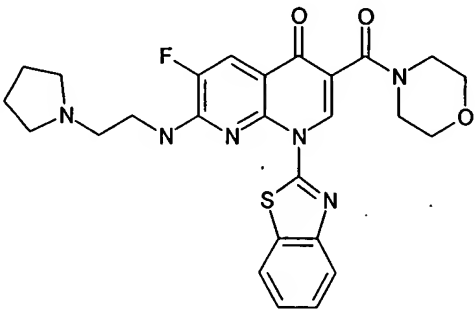
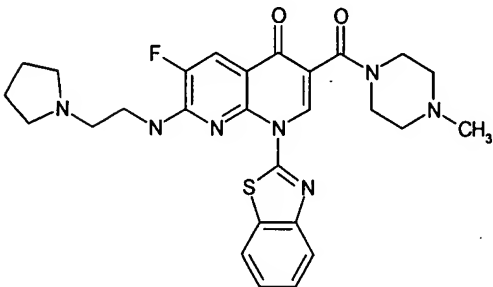
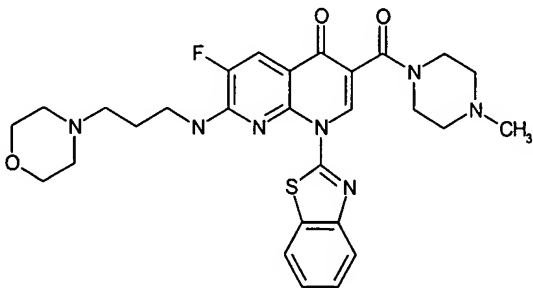
[0081] The crude benzothiazole ester was dissolved in acetic acid (10 ml) and 12M HCl (10 ml) and heated to 50°C for 3 h. The resulting solution was cooled and evaporated until a solid formed and was collected. 7-chloro-6-fluoro-1,4-dihydro-4-oxo-1-(thiazol-2-yl)-1,8-naphthyridine-3-carboxylic acid (5a) was used without further purification.

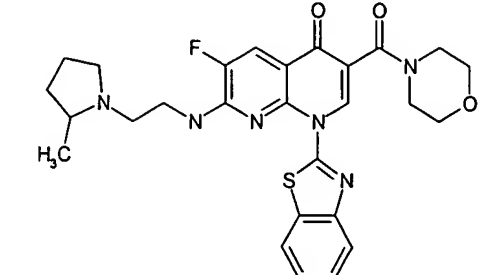
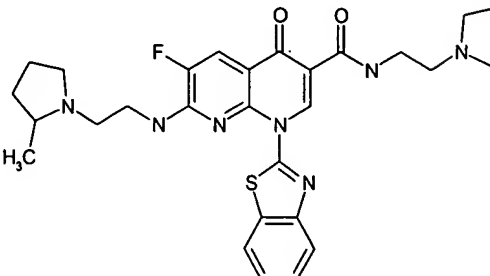
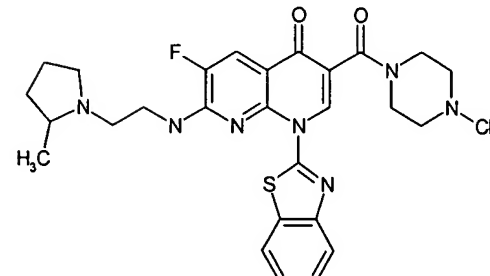
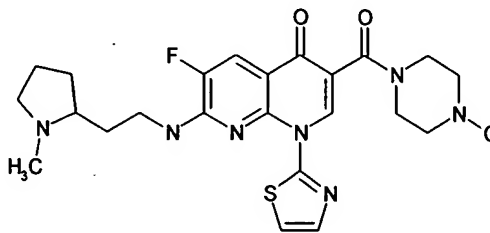
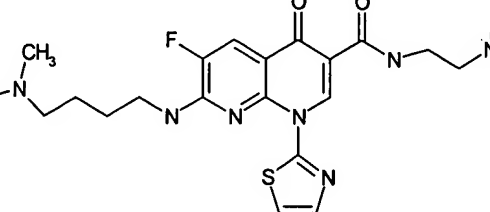
[0082] Products were then reacted with amines using the following general procedure:



[0083] To a series of solutions of the fluoroacid (0.5 mmol) in NMP (3.6 mL) was added the amines  $NHR_1R_2$  (0.5-2.0 mmol) at room temperature. The vessel were sealed and heated on a  $90^\circ C$  hotplate with constant stirring for 1-2 hours until the reactions were determined to be complete by HPLC/MS analysis. The reaction mixtures were allowed to cool to room temperature and water was added (20 mL). The resulting precipitates were collected by vacuum filtration and dried under vacuum. In cases where 1.0 equivalent of amine was used, the resulting reaction mixtures were used in the next step "as is". The resulting solids or solutions were treated with HBTU (2.5 eq.) and DIEA in 3.6 mL NMP and allowed to stir for 30 minutes at room temperature under an inert atmosphere. These solutions were added to a series of amines  $NHR_3R_4$  (2.5 equivalents) in a 96 well format (Whatman Uniplate, 2mL) and allowed to react for 2 hours. Methanol was then added (50-100  $\mu L$ ) and the plate was filtered (Whatman Unifilter Polypropylene). The resulting liquids were directly chromatographed on reverse HPLC (Waters Xterra 19X50 mm) with mass directed collection (Micromass ZQ, Waters FCII). The fractions were analyzed for purity (MS TIC, UV) and dried by vacuum evaporation (Savant), with an average yield of 5-10 mg). Structures, molecular weights, and formulas for final products are set forth in Table 1.

Table 1

ID	Structure	Formula Structure	mol weight Structure
1.		C30H37FN8O2S	592.7442
2.		C26H27FN6O3S	522.6059
3.		C27H30FN7O2S	535.6483
4.		C28H32FN7O3S	565.6747

ID	Structure	Formula Structure	mol weight Structure
5.		C27H29FN6O3S	536.633
6.		C29H34FN7O2S	563.7024
7.		C28H32FN7O2S	549.6753
8.		C24H30FN7O2S	499.6148
9.		C24H32FN7O2S	501.6307

ID	Structure	Formula Structure	mol weight Structure
10.		C23H30FN7O2S	487.6037
11.		C25H33FN8O2S	528.6566
12.		C23H27FN6O3S	486.5724
13.		C25H32FN7O2S	513.6419

[0084] It is understood that the foregoing detailed description and accompanying examples are merely illustrative, and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, formulations and/or methods of use of

the invention, may be made without departing from the spirit and scope thereof. U.S. patents and publications referenced herein are incorporated by reference.